Prognostic role of Epidermal growth factor receptor variant III (EGFRvIII) positivity in EGFR-amplified primary and recurrent glioblastomas

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Epidermal growth factor receptor variant III (EGFRvIII) positivity in *EGFR*-amplified glioblastomas: Prognostic role and comparison between primary and recurrent tumors

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EGFR amplification and EGFRvIII in glioblastoma

Key words

Glioblastoma, *EGFR* amplification, EGFRvIII, *EGFR* single nucleotide variants, *MGMT* promoter methylation, prognosis, tumor recurrence

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Conflicts of interest

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Abstract

Purpose: Approximately 40% of all glioblastomas have amplified the epidermal growth factor receptor (*EGFR*) gene and about half of these tumors express the EGFRvIII variant characterized by an in-frame deletion of exons 2-7. The prognostic role of EGFRvIII in *EGFR*-amplified glioblastoma patients and changes in EGFRvIII expression in recurrent versus primary glioblastomas remain controversial, but such data are highly relevant for the clinical evaluation of EGFRvIII-targeted therapeutic agents.

Experimental design: EGFR-amplified glioblastomas from 106 patients treated according to standard of care were assessed for EGFRvIII positivity. Changes in *EGFR* amplification and EGFRvIII status from primary to recurrent glioblastomas were evaluated in 40 patients with *EGFR*-amplified tumors, including 25 EGFRvIII-positive cases, and 33 patients with *EGFR*-non-amplified tumors. *EGFR* single nucleotide variants (SNVs) were additionally assessed in 27 patients. Data were correlated with outcome including validation in an independent cohort of 150 glioblastoma patients from The Cancer Genome Atlas (TCGA) consortium.

Results: Sixty of 106 *EGFR*-amplified glioblastoma patients had EGFRvIII-mutant tumors (56.6%). EGFRvIII positivity was not associated with different progression-free survival (HR=0.91, 95%CI 0.61-1.36, p=0.644) or overall survival (HR=1.05, 95%CI 0.70-1.58, p=0.798). EGFRvIII status was unchanged at recurrence in 35 of 40 patients with *EGFR*-amplified primary tumors (87.5%). In four patients, EGFRvIII positivity was lost at recurrence while one recurrent glioblastoma gained EGFRvIII positivity. None of 33 *EGFR*-non-amplified glioblastomas acquired *EGFR* amplification or EGFRvIII at recurrence. *EGFR* SNVs were frequent in *EGFR*-amplified tumors, but were not linked to survival.

Conclusions: EGFRvIII and *EGFR* SNVs are not prognostic in *EGFR*-amplified glioblastoma patients. *EGFR* amplification is retained in recurrent glioblastomas. Most EGFRvIII-positive glioblastomas maintain EGFRvIII positivity at recurrence. However, EGFRvIII expression may change in a subset of patients at recurrence, thus repeated biopsy with reassessment of

EGFRvIII status is recommended for recurrent glioblastoma patients to receive EGFRvIII-targeting agents.

Abstract word count: 294 words

Statement of translational relevance

The epidermal growth factor receptor (*EGFR*) gene is amplified in approximately 40% of glioblastomas. About half of the *EGFR*-amplified tumors are positive for the tumor-specific EGFRvIII deletion variant and *EGFR* single nucleotide variants (SNVs) are also commonly associated with *EGFR* amplification. Various novel therapeutic agents targeting overexpressed EGFR or EGFRvIII proteins are currently being developed. The present study indicates that positivity for EGFRvIII and presence of one or more *EGFR* SNVs are not prognostic in patients with *EGFR*-amplified glioblastomas. In addition, we show that *EGFR* amplification is generally maintained between primary and recurrent glioblastomas. However, the EGFRvIII status in *EGFR*-amplified glioblastomas may change upon tumor recurrence in a subset of patients, suggesting a role for reassessment of the EGFRvIII status in recurrent glioblastoma patients to receive an EGFRvIII-targeting treatment.

Abbreviations

CI, confidence interval; CNS, central nervous system; EGFR, epidermal growth factor receptor; EGFRvIII, EGFR variant III; GGN, German Glioma Network; HR, hazard ratio; IDH, isocitrate dehydrogenase; IHC, immunohistochemistry; KPS, Karnofsky Performance Score; MGMT, O6-methylguanine-DNA methyltransferase; MSP, methylation-speficic PCR; NGS, next generation sequencing; OS, overall survival; PD, progressive disease; PFS, progression-free survival; PT, primary tumor; RT, recurrent tumor; RTK, receptor tyrosine kinase; RT-PCR, reverse transcriptase-PCR; SNV, single nucleotide variant; TCGA, The Cancer Genome Atlas; TMZ, temozolomide; TMZ/radiotherapy→TMZ, radiotherapy with concomitant and maintenance TMZ.

Introduction

The epidermal growth factor receptor (*EGFR*) gene is the most commonly amplified and overexpressed proto-oncogene and a frequent mutational target in glioblastoma (for reviews see 1, 2). *EGFR* gene amplification is detectable in approximately 40% of all glioblastomas (3-5) and is particularly common in the classic or receptor tyrosine kinase (RTK) type 2 molecular subtype of isocitrate dehydrogenase (IDH)-wildtype glioblastoma (6). Approximately 50% of *EGFR*-amplified glioblastomas do not only amplify and overexpress the wildtype *EGFR* gene but additionally carry a tumor-specific deletion variant (EGFRvIII) that is characterized by an in-frame deletion of exons 2-7 (7, 8). This particular rearrangement results in overexpression of a truncated receptor protein that lacks major parts of the extracellular domain, is unable to bind its ligands and is constitutively active, thus constituting a prototypic oncoprotein (for review see 9). Futhermore, the EGFRvIII protein carries a unique peptide sequence generated by the fusion of exons 1 and 8 which may serve as a tumor-specific target for anti-EGFRvIII immunotherapy approaches including antibody-based approaches, genetically modified T cells, as well as peptide-based vaccination strategies (for review see 10).

Standardization of detection and quantification of *EGFR* amplification and EGFRvIII mutation in routinely processed tumor tissues remain challenging. Few studies suggested that EGFRvIII may occur in the absence of *EGFR* amplification in minor subsets of anaplastic astrocytomas and glioblastomas (11, 12). However, most studies indicate a close link between *EGFR* amplification and EGFRvIII expression (1, 9, 13), which both are nowadays considered as typical alterations in IDH-wildtype glioblastomas (6, 14, 15).

The prognostic role of *EGFR* amplification and EGFRvIII mutation in glioblastoma patients remains controversial. Individual studies suggested that these alterations are associated with shorter overall survival (OS) among anaplastic astrocytoma patients (12) and glioblastomas patients (11), while other authors found a prognostically favorable role of EGFRvIII positivity in glioblastoma patients (16). Yet other studies, including previous publications of the

German Glioma Network (13, 17), as well as a recent meta-analysis of eight publications (18), did not confirm a distinct prognostic role of *EGFR* amplification and EGFRvIII positivity, although a trend towards decreased long-term survival with EGFRvIII-positive glioblastoma has been reported (13, 19, 20).

Because of the ongoing development and clinical evaluation of various targeted treatment strategies directed against wildtype EGFR and/or against EGFRvIII (10), including e.g. peptide-based vaccines such as rindopepimut (21) and monoclonal antibody-based immunotoxins such as ABT-414 (22), a better understanding of the biological role and the prognostic significance of *EGFR* amplification and EGFRvIII status in glioblastoma is urgently needed. In particular, the prognostic role of EGFRvIII within the population of patients with *EGFR*-amplified glioblastoma has not been conclusively determined. Moreover, although most novel targeted agents are initially being tested in patients with progressive glioblastoma after failure of standard therapy consisting of local fractionated radiotherapy with concomitant and maintenance chemotherapy with temozolomide (TMZ/radiotherapy→TMZ), data regarding the expression and role of EGFRvIII in the recurrent setting are sparse and in part conflicting (23, 24). Accordingly, we explored the prognostic role of EGFRvIII expression among newly diagnosed patients with *EGFR*-amplified glioblastomas and determined the stability of *EGFR* amplification and the EGFRvIII status at recurrence following standard of care treatment. In a subset of patients, we performed targeted sequencing of the *EGFR* gene to evaluate additional *EGFR* sequence alterations for associations with *EGFR* gene amplification and prognosis, as well as for changes upon tumor recurrence.

Patients and Methods

Patients

The present study was based on 106 patients with newly diagnosed *EGFR*-amplified, IDH-wildtype glioblastomas. A total of 52 patients underwent second surgery for recurrent tumors.

From 40 of these patients, matched tissue specimens were available from primary and recurrent tumors (Supplementary Table 1). In addition, 33 primary and recurrent tumor pairs from patients with newly diagnosed, IDH-wildtype and *EGFR*-non-amplified tumors were studied (Supplementary Table 2). The patients were identified in the central database of the German Glioma Network (GGN) or the database of the Central Nervous System (CNS) tumor tissue bank at the Department of Neuropathology, Heinrich Heine University, Düsseldorf, Germany. They included 74 patients from the previous GGN study on the assessment of EGFRvIII expression in glioblastoma tissues obtained at first operation (13). The EGFRvIII status of the 106 primary tumors with *EGFR* amplification was determined by immunohistochemistry in 97 (91.5%) patients, by RT-PCR analysis in 88 (83.0%) patients and by both methods in 79 (74.5%) patients. For determination of changes in *EGFR* amplification and protein expression as well as EGFRvIII positivity between primary and recurrent glioblastomas, we additionally investigated recurrent glioblastoma tissue samples from 73 of the 85 patients who had second surgery (median interval between primary and recurrent resections in the 85 patients: 9.1 months, range: 3.3 – 42.7 months). In 12 patients, tissue from second surgery was either not available or not sufficient for further analyses. The other 73 patients included 25 patients with *EGFR*-amplified and EGFRvIII-positive primary tumors, 15 patients with *EGFR*-amplified but EGFRvIII-negative primary tumors, and 33 patients with *EGFR*-non-amplified tumors. Histology of the tumors was centrally reviewed and confirmed to correspond to glioblastoma World Health Organization (WHO) grade IV, originally based on the WHO classification of central nervous system tumors 2007 (25). All cases were later on shown to correspond to glioblastoma, IDH-wildtype, WHO grade IV according to the WHO classification of central nervous system tumors 2016 (26). Patients gave their written informed consent for participating in the German Glioma Network and the use of their tissue samples and clinical data for research purposes. The present study was approved by the institutional review board of the Medical Faculty, Heinrich Heine University, Düsseldorf, Germany (study number 4700).

Extraction of nucleic acids

DNA and RNA were extracted from frozen tumor tissue samples either by ultracentrifugation (27, 28) or by using the JETQUICK Tissue DNA Spin Kit (Genomed, Loehne, Germany) and the RNeasy Mini Kit (Qiagen, Hilden, Germany). DNA and RNA preparation from formalin-fixed and paraffin-embedded (FFPE) samples was performed with the QIAmp DNA FFPE Tissue Kit (Qiagen) and the RNeasy FFPE Kit (Qiagen).

PCR-based detection of EGFR amplification and EGFRvIII rearrangement

Detection of *EGFR* gene amplification by real-time PCR was performed as reported (29-31). The following primers were used for *EGFR*: EGFR-F (5'-cactgcctcatctctcaccatc-3') and EGFR-R (5'-gactcaccgtagctccagac-3'). Primers for the *WI-3306* locus on 2q that served as reference locus were: WI-3306-F (5'-catgactgcgagcccaagatg-3') and WI-3306-R (5'-caggtggtgtcatcagaatcag-3'). For each tumor, the target:reference gene ratio was normalized to the target:reference gene ratio of human normal brain DNA using the comparative $\Delta\Delta C_T$ method. As positive control for *EGFR* gene amplification, we used tumor DNA extracted from a glioblastoma with known *EGFR* amplification. Only tumors showing a normalized target:reference gene ratio ≥ 3 were considered as showing *EGFR* gene amplification.

Detection of EGFRvIII positivity by qualitative RT-PCR has been reported elsewhere (13). The following primers located in *EGFR* exons 1 and 8 were used to generate products of 92 bp for the *EGFRvIII* and 893 bp for *EGFR* wildtype (wt) mRNA sequences: EGFR-Ex1-F: GAGTCGGGCTCTGGAGGAAA; EGFR-Ex8-R: CCATCTCATAGCTGTCGGGCC. The EGFRwt product of 893 bb, however, was only detected in case when high-molecular weight RNA extracted from frozen tissue samples was used for cDNA generation. In case of RNA extracted from FFPE tissue samples, this fragment was usually not detectable due to RNA degradation. Therefore, we used additional primers located in exon 1 (as described above) and exon 2 (EGFR-Ex2-R: CAGTTATTGAACATCCTCTGGAG) generating a product of 111 bp representing *EGFR* wildtype sequences (Figure 1). PCR was performed with HotStarTaq-Polymerase (Qiagen) for 15 min at 95°C, followed by 40 cycles for 30 sec at 95°C, 30 sec at

60°C (EGFR-Ex1-F, EGFR-Ex8-R) or 58°C (EGFR-Ex1-F, EGFR- Ex2-R) and 1 min at 72°C (13) (Figure 1).

Immunohistochemistry for EGFR and EGFRvIII protein

Immunohistochemistry was performed as reported (13). For antigen retrieval, rehydrated sections were treated in 10 mM citrate buffer at pH 6.0 (for staining with E30 or 6549 antibodies) or at pH 9.0 (for staining with DAK-H1-WT) for 20 min in a steamer. Sections were immunostained either with the mouse monoclonal antibody DAK- H1-WT (Dako, Copenhagen, Denmark) that detects only the wildtype EGFR protein (antibody dilution: 1:200), a rabbit polyclonal antiserum (lot #6549, Celldex, Needham, MA) that exclusively detects the EGFRvIII protein (antibody dilution: 1:5000), or the monoclonal mouse antibody E30 (Dako) that detects both wildtype EGFR and EGFRvIII proteins (antibody dilution: 1:200). Immunoreactivity for wildtype EGFR (DAK-H1-WT, E30) was semiquantitatively scored as follows: -, negative; +, weakly positive; ++, moderately positive; +++, strongly positive (32). EGFRvIII immunoreactivity was classified as positive when immunoreactive tumor cells were detectable (irrespective of the fraction of positive tumor cells) or as negative when immunoreactive tumor cells were absent (Figure 2). Evaluation of the immunohistochemical stainings was jointly performed by two experienced neuropathologists (J.F. and G.R.).

Analysis for IDH mutation

All tumors were screened for IDH1-R132H mutation using immunohistochemistry with the monoclonal antibody clone H09 (Dianova, Hamburg, Germany) (33). Tumors from patients younger than 55 years of age were additionally investigated for other *IDH1* or *IDH2* mutations using Sanger sequencing or pyrosequencing as reported (29, 34) and recommended in the WHO classification 2016 (26).

O6-methylguanine-DNA methyltransferase (MGMT) promoter methylation analysis

The *MGMT* promoter methylation status was determined for all tumor samples using methylation-specific PCR (MSP) analysis as reported (35). Tumor DNA was treated with sodium bisulfite using the EZ DNA Methylation-Gold Kit™ (HISS Diagnostics, Freiburg, Germany). DNA from the A172 glioma cell line (obtained from American Type Culture Collection, ATCC) was used as positive (methylated) control while peripheral blood leukocyte DNA served as negative (unmethylated) control. In addition, a no template DNA control was run with each experiment.

Targeted sequencing of the EGFR coding sequence

In a subset of 27 primary and recurrent paired glioblastoma samples, ~~pairs (~~including 13 pairs with and 14 pairs without *EGFR* amplification~~)~~, we performed targeted next generation sequencing (NGS) of the *EGFR* coding sequence employing an amplicon-based approach for a pre-defined glioma gene panel and the Ion Proton™ sequencing platform as reported (31). In total, 59 amplicons covering the entire *EGFR* coding region from exon 1 to exon 28 were amplified from tumor DNA and sequenced. Evaluation of the NGS data for sequence variations and copy number alterations was performed as described in detail elsewhere (31).

Evaluations involving The Cancer Genome Atlas (TCGA) data sets

We retrieved data from 150 IDH-wildtype glioblastoma patients with available information on *EGFR* amplification and *EGFR* single nucleotide variants in the TCGA glioblastoma dataset (https://portal.gdc.cancer.gov/projects/TCGA-GBM). For a subset of these patients (n = 66) the EGFRvIII status was additionally available. Information concerning ~~temozolomide therapy, overall survival,~~ age at diagnosis, gender, *MGMT* promotor methylation status, ~~and~~ IDH mutation status, temozolomide therapy and OS were retrieved from the respective TCGA publications (14, 15). *EGFR* copy number data (GISTIC-scores) and *EGFR* mutation calls (SNVs) from the TCGA-GBM dataset were downloaded via the cBio Cancer Genomics Portal (www.cbioportal.org) using the open-source R package “cgsdr” (version 1.2.5) and the

statistical computing language R (version 3.3.2). Each case with at least one SNV in *EGFR* was classified as "SNV in *EGFR*". *EGFR* amplification was assumed if the GISTIC score was 2. Cases with EGFRvIII allele frequencies (AF) > 0.01 as reported by Brennan et al. (14) were regarded as EGFRvIII-positive.

Statistical analyses

In the GGN cohort, progression-free survival (PFS) was calculated from the day of first surgery until tumor progression, death, or end of follow-up. ~~Overall survival (~~OS~~)~~ was calculated from the day of first surgery until death or end of follow-up. Kaplan-Meier survival curves and Logrank test as well as Cox regression analyses were used for univariate and multivariate analyses of survival data. Statistical analyses were performed with IBM SPSS Statistics version 24.0 or the R-package "survival" (version 2.40-1). To test for associations between EGFR-SNV status and *EGFR* amplification or EGFRvIII positivity, we used the Fisher's exact test from the basic R-package "stats" (version 3.3.2).

Results

Prognostic significance of EGFRvIII in EGFR-amplified glioblastoma

The present study is based on 106 patients with *EGFR*-amplified, IDH-wildtype glioblastomas documented in the GGN central data base. Clinical characteristics, treatment and outcome of this patient cohort are summarized in Table 1 according to EGFRvIII status. EGFRvIII positivity was detetected in 60 of the 106 tumors (56.6%). EGFRvIII expression was detetected by immunohistochemistry in 49 of 97 tumors investigated (50.5%), while RT-PCR for EGFRvIII was positive in 50 of 88 tumors investigated (56.8%) (Supplementary Table 1). Among the 79 tumors evaluated for EGFRvIII by immunohistochemistry and RT-PCR, a total of 39 tumors were EGFRvIII-positive by both methods (49.4%) (Figures 1 and 2). All tumors

with immunohistochemical positivity for EGFRvIII were also EGFRvIII-positive by RT-PCR, whereas 9 tumors lacked immunohistochemical EGFRvIII positivity but showed positive results by RT-PCR, thus indicating a higher sensitivity of RT-PCR analysis or suppression of translation or both (Figures 1 and 2, Supplementary Table 1). *EGFR*-amplified glioblastomas generally showed strong and widespread immunopositivity with antibodies detecting wildtype EGFR proteins or both wildtype EGFR and EGFRvIII (Figure 2). In contrast, EGFRvIII immunopositivity was frequently restricted to subpopulations of tumor cells, with sometimes striking regional distribution (Supplementary Figure 1).

Patients with EGFRvIII-positive tumors were slightly older (p=0.081) and less often had a high KPS (p=0.253) than patients with *EGFR*-amplified but EGFRvIII-negative glioblastomas (Table 1). However, PFS and OS did not differ in patients with *EGFR*-amplified glioblastomas stratified according to EGFRvIII status (Figure 3A-B). Hazard ratios (HR) with regard to PFS and OS were determined (HR=0.91, 95% CI 0.61-1.36, p=0.644; HR=1.05, 95% CI 0.70-1.58, 0.798). HR were observed in the same order after adjustment for *MGMT* promoter methylation and first-line therapy (data not shown). EGFRvIII status was not associated with *MGMT* promoter methylation status (p=0.265). *MGMT* promoter methylation, but not EGFRvIII expression, was associated with longer OS in the 77 patients treated with radiotherapy and temozolomide chemotherapy (Supplementary Figure 2A-B). Further stratification of these patients according to EGFRvIII status and *MGMT* promoter methylation revealed that *MGMT* promoter methylation was associated with longer OS in patients with EGFRvIII-negative tumors but was not prognostic in patients with EGFRvIII-positive glioblastomas (Supplementary Figures 2C-D).

Changes in EGFRvIII expression between paired primary and recurrent glioblastomas

We investigated 40 glioblastoma patients with *EGFR*-amplified primary tumors who were treated by second surgery at progression and from whom representative tissue sections with viable tumor tissue were available from both primary and recurrent tumors. Of these, 25 patients had EGFRvIII-positive primary tumors as detected by RT-PCR,

immunohistochemistry or both methods (Supplementary Table 1). Important patient characteristics are summarized in Table 1. Compared with patients who did not receive second surgery, patients with second surgery had more often received a gross total resection (28/44 patients versus 19/47 patients, p=0.027) and radiotherapy with concomitant and maintenance temozolomide chemotherapy (TMZ/radiotherapy→TMZ) as first-line treatment (43/52 patients versus 34/54 patients, p=0.023), but clinical characteristics were otherwise similar. One of the initially 15 EGFRvIII-negative tumors (by both immunohistochemistry and RT-PCR) became EGFRvIII-positive at recurrence by RT-PCR but not by Immunohistochemistry (Figure 1, patient 79). Among the recurrent tumors of the 25 initially EGFRvIII-positive patients undergoing second surgery, 21 patients (84%) retained EGFRvIII expression as detected by immunochemistry (15 patients, 71%), RT-PCR (18 patients, 86%) or both methods (12 patients, 57%) (Figures 1 and 2, Supplementary Table 1). In four patients, recurrent glioblastomas lost EGFRvIII positivity as determined by both methods for three patients and by immunohistochemistry for one patient (Figures 1, 2 and 4, Supplementary Table 1). There were overall no major differences in clinical characteristics as well as PFS, OS, and post-recurrence survival between patients who did or did not receive a second operation when each group was stratified according to EGFRvIII status (Supplementary Figure 3).

To evaluate whether EGFRvIII-negative and *EGFR*-non-amplified glioblastomas may newly acquire EGFRvIII positivity and/or *EGFR* amplification, we additionally investigated paired primary and recurrent glioblastoma tissues samples from 33 patients with *EGFR*-non-amplified primary glioblastomas. Recurrent tumors in none of these patients demonstrated newly acquired *EGFR* amplification or EGFRvIII positivity (Supplementary Table 2).

Association of EGFR SNVs with EGFR amplification, tumor recurrence and OS~~patient survival~~

To assess a role of ~~other~~ *EGFR* sequence alterations other than EGFRvIII in ~~glioblastoma~~ recurrent glioblastoma~~ce~~, we performed ~~next generation sequencing (~~NGS~~)~~ of the *EGFR*

coding sequence in 27 paired samples of primary and recurrent glioblastomas, including 13 paired samples with *EGFR* gene amplification. The detected *EGFR* SNVs generally corresponded to missense mutations, including several mutations previously reported in other studies as summarized in Supplementary Table 3. Since we performed NGS analyses for individual exons at the DNA level, we could not detect larger rearrangements and/or deletions previously reported (14), except for EGFRvIII. However, sensitivity of EGFRvIII detection by gene panel NGS was lower than with RT-PCR or immunohistochemistry (8/10 investigated tumors with EGFRvIII positivity), in line with published data (31). *EGFR* SNVs were found in eight of 13 *EGFR*-amplified primary tumors (including seven of 11 EGFRvIII-positive tumors and one of two EGFRvIII-negative tumors), but only in one of 14 *EGFR*-non-amplified primary tumors ($p<0.01$, Fisher's exact test). Presence of an *EGFR* missense mutation was not associated with distinct OS in our cohort of 27 patients (Supplementary Figure 4).

In 11 of 14 GGN patients with one or more *EGFR* SNVs detected by NGS, the individual SNVs identified in the primary tumor were retained in the respective recurrence. In three patients, however, *EGFR* SNVs were lost from primary to recurrent tumor, while seven patients showed *EGFR* SNVs in their recurrent tumors that were not detectable in the matched primary tumors (Supplementary Table 3).

Validation studies based on TCGA glioblastoma patients

In line with the findings in the GGN cohort, interrogation of the TCGA database showed no OS difference in a cohort of 150 IDH-wildtype glioblastoma patients treated with temozolomide when patients were stratified according to *EGFR* amplification status or according to the presence of at least one EGFR SNV (Supplementary Figure 5A-B, Supplementary Table 5). Presence of *EGFR* SNVs was significantly associated with *EGFR* gene amplification in the TCGA cohort (25/79 *EGFR*-amplified glioblastomas *versus* 6/71 *EGFR*-non-amplified tumors, $p < 0.001$). Within the group of 79 patients with *EGFR*-amplified glioblastomas, IDH-wildtype, additional presence of *EGFR* SNVs was not associated with

~~overall survival~~OS (Supplementary Figure 5C). In the 66 cases with available information on EGFRvIII status, there was no association between the presence of an *EGFR* SNV and EGFRvIII positivity (3 SNV in *EGFR*-positive / 12 EGFRvIII-positive tumors versus 14 SNV in *EGFR*-positive / 54 EGFRvIII-negative tumors, p=0.95). Finally, EGFRvIII positivity was not associated with distinct OS in the subgroup of 32 TCGA patients with *EGFR*-amplified tumors and available information on EGFRvIII status (Supplementary Figure 5D).

Discussion

Interest in the biological role and the clinical significance of *EGFR* amplification and other EGFR alterations, in particular the constitutively active EGFRvIII deletion variant, in glioblastoma has increased over recent years. Accumulating preclinical evidence has attributed an important function of EGFRvIII-expressing glioblastoma cells in driving tumor heterogeneity and progression by promoting glioma cell proliferation, invasion, angiogenesis, stemness and therapy resistance in different model systems (36-43). In addition, several therapeutic approaches targeting overexpressed wildtype EGFR protein or specifically EGFRvIII have already entered, or are about to enter clinical evaluation, including peptide-based vaccines (44-46), chimeric antigen receptor (CAR) T cells (47, 48), as well as anti-EGFR antibody-based approaches (22, 49, 50).

Previous studies reported on conflicting results concerning the prognostic role of EGFRvIII, with a meta-analysis of eight published studies indicating no obvious association of *EGFR* amplification or EGFRvIII positivity with survival of glioblastoma patients (18). Our present study confirms these data and additionally shows that the presence of EGFRvIII is not prognostic among patients with *EGFR*-amplified glioblastoma treated according to current standard of care (Table 1, Figure 1).

In our patient cohort, *MGMT* promoter methylation was prognostic in patients treated with radiochemotherapy and was particularly associated with longer OS in the subgroup of

patients with EGFRvIII-negative tumors. In contrast, *MGMT* promoter methylation was not prognostic in the subgroup of patients with EGFRvIII-positive glioblastomas. However, we could not confirm the suggestion of a prognostic interaction between EGFRvIII expression and *MGMT* promoter methylation in a published data set of 13 patients with EGFRvIII positive tumors and available *MGMT* promoter methylation status (16), and in the large ACT IV phase 3 dataset (51).

In line with previous studies, our immunohistochemical findings confirm that EGFR wildtype protein expression is strong and widespread in *EGFR*-amplified glioblastomas (11, 17, 52). We did not assess regional heterogeniety of *EGFR* gene amplification. However, previous studies reported that *EGFR* amplification may be restricted to subpopulations of tumor cells in rare cases of glioblastomas with amplification of *EGFR* and *PDGFRA* (53, 54). With respect to EGFRvIII expression, our data demonstrate that EGFRvIII immunopositivity shows marked regional heterogeneity and is often restricted to subpopulations of tumor cells in glioblastomas, thus confirming previous findings in the GGN patient cohort (17) and in several independent studies (39, 42, 43, 52, 55).

We also addressed the clinically relevant question whether *EGFR* amplification and EGFRvIII expression may change from primary to recurrent glioblastomas following standard therapy. Montano et al. (56) reported on a trend towards lower expression of EGFRvIII in recurrent as opposed to corresponding primary glioblastomas based on the analysis of 13 patients. Van den Bent et al. (23) investigated matched pairs of primary and recurrent glioblastomas from 55 patients, including 23 patients with tumors demonstrating high-copy *EGFR* amplification, and found that the *EGFR* amplification status remained stable in 46 of 55 patients (84%). EGFRvIII mRNA expression as determined by RT-PCR was found to be lost from primary to recurrent tumors in 7 of 15 initially EGFRvIII-positive tumors. In contrast, other authors detected no loss of EGFRvIII positivity upon tumor recurrence following standard radiochemotherapy in 15 of 15 patients with EGFRvIII positive glioblastomas, while 16 of 16 patients treated with anti-EGFRvIII vaccination demonstrated no more EGFRvIII expression upon tumor recurrence (24). In our study, we evaluated *EGFR* amplification and

expression at the DNA and protein levels, as well as EGFRvIII expression at the mRNA and protein levels. Thereby, we clearly demonstrated that *EGFR* amplification and the associated overexpression of EGFR protein in IDH-wildtype glioblastomas generally remain stable upon recurrence following standard therapy. In addition, investigation of 33 patients with *EGFR* non-amplified primary glioblastomas, IDH-wildtype, did not reveal a single patient whose tumor newly acquired *EGFR* amplification upon recurrence. EGFRvIII positivity persisted from primary to recurrent glioblastomas in 21 of 25 patients (84 %) with initially EGFRvIII-positive tumors. However, glioblastomas in four patients had lost their initial EGFRvIII positivity upon recurrence while a single patient with an *EGFR*-amplified glioblastoma showed EGFRvIII positivity only in the recurrent tumor (Figure 4). The reason for the lower rate of tumors that lost EGFRvIII positivity upon recurrence in our cohort, as compared to the study of van den Bent et al. (23), are unclear. We carefully checked by histological review that all recurrent tumor specimens included in our series indeed contained vital cellular tumor tissue and not just reactive and reactive changes due to cytotoxic therapy, in particular radiotherapy. Thereby, we excluded false-negative findings due to insufficient tumor cell content and radiation necrosis. Thus, available data (23, present study) suggest that EGFRvIII expression may be lost following standard radiochemotherapy in a subset of patients, challenging the significance of previous observations reporting on loss of EGFRvIII positivity specifically in recurrent glioblastomas after peptide-based vaccinitation against EGFRvIII but not after radiochemotherapy (24). Moreover, the finding that EGFRvIII expression is more commonly reduced or lost than increased or newly gained upon glioblastoma recurrence suggests a limited role of EGFRvIII in driving radiochemotherapy resistance and disease progression in glioblastoma patients, as suggested by studies in preclinical glioma models (36, 57).

Several studies have reported on various other *EGFR* sequence alterations than EGFRvIII in glioblastomas, including SNVs as well as larger rearrangements/deletions affecting the extracellular or intracellular domains (8, 14, 58-61). We therefore additionally investigated a subset of 27 pairs of primary and recurrent glioblastomas for other *EGFR* gene mutations

using targeted next generation sequencing of tumor DNA. Thereby, we identified various *EGFR* SNVs leading to missense mutations, especially in tumors with *EGFR* gene amplification, thus corroborating data from other groups reporting on a frequent coincidence of *EGFR* amplification with other *EGFR* mutations (14, 58-60). Neither the results in our GGN cohort nor data from the TCGA consortium revealed evidence for an independent prognostic role of *EGFR* SNVs in patients treated according to the current standard of care. This finding reflects that *EGFR* SNVs are closely linked to *EGFR* amplification, which lacks prognostic significance in IDH-wildtype glioblastoma patients (13, 17, 18). Moreover, TCGA data do not support a prognostic role of *EGFR* SNVs among patients with *EGFR*-amplified glioblastomas (Supplementary Figure 54C).

We also investigated whether *EGFR* SNVs may change from primary to recurrent glioblastomas in individual patients. Similar to the findings for EGFRvIII, we observed that most of the *EGFR* SNVs detected in primary glioblastomas remain stable at recurrence. However, in three patients point mutations detected in primary tumors were lost upon recurrence while novel *EGFR* mutations turned up in recurrent glioblastomas of seven patients. These findings would be in line with a branched tumor evolution model, suggesting that recurrent glioblastomas following therapy may develop from minor subclones of the respective primary tumor. In addition, it is possible that *EGFR* point mutations detected exclusively in recurrent tumors are induced by therapy, in particular in case of C-G to T-A transitions that are known to be related to DNA-alkylating treatment with temozolomide (62). These issues require further analyses by more comprehensive molecular investigations of longitudinal biopsies in a larger cohort of glioblastoma patients.

In summary, our study shows that EGFRvIII is not prognostic in *EGFR*-amplified glioblastoma patients. *EGFR* amplification in glioblastomas is often associated with additional genetic changes including EGFRvIII and various SNVs. Upon tumor recurrence, the *EGFR* amplification status of the primary tumor is generally retained and the majority of EGFRvIII-positive glioblastomas maintain EGFRvIII positivity at recurrence. However, EGFRvIII expression may change in a subset of patients at recurrence. Thus, in patients with recurrent

glioblastoma who are evaluated for EGFRvIII-directed therapy approaches, either on compassionate use or within clinical trials, re-assessment of the EGFRvIII status should be performed using recurrent glioblastoma tissue specimens to assure that the therapeutic target is still expressed on the tumor cells.

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Kommentiert [WM1]: Ref 51 ist in press und Ref 46 brauchen wir dann nicht mehr

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Legends to the Figures

Figure 1. Agarose gel electrophoresis images of results obtained by RT-PCR for EGFR wildtype (wt) and EGFRvIII mRNA expression in pairs of primary tumors (PT) and recurrent tumors (RT) of five selected patients (1, patient 97; 2, patient 81; 3, patient 99; 4, patient 79; 5, patient 100) with *EGFR*-amplified glioblastomas as demonstrated by real-time PCR (not shown). Note that EGFRwt transcripts are expressed in all tumors as indicated by a 111 bp PCR product obtained by RT-PCR with primers detecting *EGFR* sequences in exons 1 and ~~exon~~ 2 (upper panel). In the lower panel. RT-PCR was performed with primers specific for *EGFR* exons 1 and ~~exon~~ 8, which amplify a 893 bp from EGFRwt transcripts and a 92 bp fragment from EGFRvIII transcripts. However, the 893-bp is obtained only when high-molecular weight RNA extracted from frozen tissue is used as template (in case of normal brain, NB), but absent when degraded RNA from FFPE material is used (in case of the tumor samples). Patient 97 expressed EGFRvIII transcripts in both primary and recurrent tumor. Patient 81 lacked EGFRvIII expression in primary and recurrent tumor. In patient 99, EGFRvIII expression in the primary tumor was lost in the recurrent tumor; in contrast, patient 79 had a clear EGFRvIII signal in the recurrent tumor that was absent in the primary tumor, while patient 100 showed a very weak, barely detectable EGFRvIII band in the primary tumor and a strong EGFRvIII signal in the recurrent tumor. C, controls: +, positive control with known EGFRvIII expression, NTC, non-template control (negative control).

Figure 2. Representative immunohistochemical results obtained in four glioblastoma patients **(A-D)**. Shown are histological features in hematoxylin-eosin (HE) stained sections and immunostainings for pairs of primary tumor (PT) and recurrent tumor (RT) from each patient. Immunohistochemical stainings were done using antibodies detecting wildtype EGFR (EGFRwt), wildtype EGFR and EGFRvIII (EGFRwt/vIII, E30) or EGFRvIII. All four depicted tumor pairs showed *EGFR* gene amplification (as demonstrated by real-time PCR) as well strong immunoreactivity for EGFRwt and EGFRwt/vIII (E30). All immunostained sections are

counterstained with hemalum. While EGFRwt expression remained similar between primary and recurrent tumors, EGFRvIII expression changed in two of the depicted pairs. **(A)** Strong immunoreactivity for EGFRwt and EGFRvIII in the primary and recurrent tumor (patient 97). **(B)** Expression of EGFRwt but not EGFRvIII in the primary and recurrent tumor (patient 81). **(C)** Expression of EGFRwt and EGFRvIII in large areas of the primary tumor but loss of positivity for EGFRvIII in the recurrent tumor (patient 99). **(D)** Only a subpopulation of EGFRvIII-positive tumor cells in the primary tumor but widespread EGFRvIII positivity in the recurrent tumor (patient 100). Bars correspond to 100 μm.

Figure 3. Survival outcome in 106 patients with *EGFR*-amplified glioblastomas, IDH-wildtype, stratified according to the EGFRvIII status. **(A)** Progression-free survival (PFS) and **(B)** overall survival (OS) show no difference according to EGFRvIII status.

Figure 4. Schematic representation of changes in EGFRvIII status in pairs of primary tumors (PT) and recurrent tumors (RT) of 73 patients with glioblastomas. Note that in 68 of the 73 tumor pairs, EGFRvIII status remained identical from primary to recurrent tumor. In 5 tumor pairs, a change was observed, including 4 instances with loss of EGFRvIII positivity upon recurrence and a single instance with newly gained EGFRvIII positivity upon recurrence. EGFR amp., *EGFR*-amplified primary tumors; EGFR not. amp., *EGFR*-non-amplified primary tumors; EGFR amp. + EGFRvIII, *EGFR*-amplified and EGFRvIII-positive primary tumors.

Table 1. Overview of the 106 patients with newly diagnosed *EGFR*-amplified glioblastoma, IDH-wildtype. Patient characteristics are stratified according to by EGFRvIII status at first surgery and according to treatment by second surgery.

	EGFRvIII-negative, all n=46	EGFRvIII-positive, all n=60	EGFRvIII-negative, no second surgery n=25	EGFRvIII-positive, no second surgery n=29	EGFRvIII-negative, second surgery n=21	EGFRvIII-positive, second surgery n=31
Age at diagnosis						
Median (years)	59	63	60	63	58	60
Range (years)	37-72	29-86	37-72	29-82	39-72	39-86
Gender						
Male	28 (60.9%)	40 (66.7%)	13 (52.0%)	20 (69.0%)	15 (71.4%)	20 (64.5%)
Female	18 (39.1%)	20 (33.3%)	12 (48.0%)	9 (31.0%)	6 (28.6%)	11 (35.5%)
KPS at diagnosis						
90-100	21 (50.0%)	17 (33.3%)	12 (48.0%)	7 (25.0%)	9 (52.9%)	10 (43.5%)
70-80	18 (42.9%)	28 (54.9%)	11 (44.0%)	16 (57.1%)	7 (41.2%)	12 (52.2%)
<70	3 (7.1%)	6 (11.8%)	2 (8.0%)	5 (17.9%)	1 (5.9%)	1 (4.3%)
No data	4	9		1	4	8
Tumor location						
Frontal	12 (26.1%)	14 (23.7%)	5 (20.0%)	7 (24.1%)	7 (33.3%)	7 (23.3%)
Temporal	11 (23.9%)	12 (20.3%)	6 (24.0%)	6 (20.7%)	5 (23.8%)	6 (20.0%)
Parietal	6 (13.0%)	9 (15.3%)	3 (12.0%)	5 (17.2%)	3 (14.3%)	4 (13.3%)
Occipital	-	4 (6.8%)	-	1 (3.4%)	-	3 (10.0%)
Not localized to one site	14 (30.4%)	14 (23.7%)	8 (32.0%)	6 (20.7%)	6 (28.6%)	8 (26.7%)
Multifocal	-	1 (1.7%)	-	1 (3.4%)	-	-
Others	3 (6.5%)	5 (8.5%)	3 (12.0%)	3 (10.3%)	-	2 (6.7%)
No data		1				1
Surgery						
Gross total resection	26 (60.5%)	21 (43.8%)	12 (50.0%)	7 (30.4%)	14 (73.7%)	14 (56.0%)
Subtotal resection (50-99%)	12 (27.9%)	22 (45.8%)	9 (37.5%)	13 (56.5%)	3 (15.8%)	9 (36.0%)
Partial resection (<50%)	3 (7.0%)	5 (10.4%)	2 (8.3%)	3 (13.0%)	1 (5.3%)	2 (8.0%)
Biopsy	2 (4.7%)	-	1 (4.2%)	-	1 (5.3%)	-
No data	3	12	1	6	2	6

Histological subtype						
Glioblastoma, IDH-wildtype	46 (100%)	59 (98.3%)	25 (100.0%)	28 (96.6%)	21 (100%)	31 (100%)
Gliosarcoma, IDH-wildtype	-	1 (1.7%)	-	1 (3.4%)	-	-
***MGMT* promoter methylation status**						
Methylated	18 (39.1%)	30 (50.0%)	9 (36.0%)	18 (62.1%)	9 (42.9%)	12 (38.7%)
Unmethylated	28 (60.9%)	30 (50.0%)	16 (64.0%)	11 (37.9%)	12 (57.1%)	19 (61.3%)
First-line treatment						
Radiotherapy alone	9 (19.6%)	13 (21.7%)	9 (36.0%)	7 (24.1%)	-	6 (19.4%)
TMZ/radiotherapy→TMZ *	34 (73.9%)	43 (71.7%)	15 (60.0%)	19 (65.5%)	19 (90.5%)	24 (77.4%)
TMZ cycles (median)	6 (1-25)	5 (1-24)	5 (2-12)	5 (1-24)	6 (1-25)	5 (1-10)
Patients with information on number of TMZ cycles	28/35	30/43	13/15	13/19	15/20	17/24
Chemotherapy alone	1 (2.2%)	-	-	-	1 (4.8%)	-
no therapy	2 (4.3%)	4 (6.7%)	1 (4.0%)	3 (10.3%)	1 (4.8%)	1 (3.2%)
PD (events)						
Salvage chemotherapy	21 (91.3%)	19 (76.0%)	9 (100.0%)	5 (83.3%)	12 (85.7%)	14 (73.7%)
Salvage radiotherapy	1 (4.3%)	2 (8.0%)	-	1 (16.7%)	1 (7.1%)	1 (5.3%)
Salvage radio-/chemotherapy	1 (4.3%)	1 (4.0%)	-	-	1 (7.1%)	1 (5.3%)
other	-	3 (12.0%)	-	-	-	3 (15.8%)
Survival						
Median PFS (months, 95% CI)	8.2 (6.5-9.9)	8.7 (6.9-10.5)	8.2 (6.4-10.0)	7.4 (5.9-8.9)	7.7 (3.0-12.4)	9.6 (8.1-11.1)
Median OS (months, 95% CI)	17.0 (10.0-23.9)	16.8 (13.6-20.1)	13.3 (11.5-15.0)	9.9 (4.0-15.7)	21.3 (17.6-25.0)	24.0 (15.6-32.5)
Follow-up range (months)	7.5-26.9	10.7-94.6	-	94.6	7.5-26.9	10.7-48.4
Alive at last follow-up	3 (6.5%)	6 (10.0%)	-	1 (3.5%)	3 (14.3%)	5 (16.1%)

Abbreviations: CI, confidence interval; KPS, Karnofsky Performance Score; PD, progressive disease; TMZ, temozolomide; TMZ/radiotherapy→TMZ, radiotherapy with concomitant and maintenance TMZ; * includes six patients who received radiotherapy and adjuvant TMZ.

Epidermal growth factor receptor variant III (EGFRvIII) positivity in *EGFR*-amplified glioblastomas: Prognostic role and comparison between primary and recurrent tumors

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Supplementary Materials

Supplementary Table 1. Overview of the individual survival times, *MGMT* promoter methylation status and results of EGFRvIII testing by immunohistochemistry (IHC) or reverse transcription-PCR (RT-PCR) in primary tumors (PT) and recurrent tumors (RT) of 106 patients with newly diagnosed *EGFR*-amplified glioblastomas, IDH-wildtype. PFS, progression-free survival; OS, overall survival; n.d., not determined; * deceased patients; ** no progression documented; § excluded for Kaplan-Meier analyses because EGFRvIII was determined at second recurrence.

Patient number	Age (years)	PFS (months)	OS (months)	MGMT promoter status	PT EGFRvIII IHC	PT EGFRvIII RT-PCR	RT EGFRvIII IHC	RT EGFRvIII RT-PCR
1	46	16.0	23.1*	unmethylated	negative	negative	-	-
2	63	44.9	72.8*	methylated	negative	n.d.	-	-
3	66	1.5**	6.2*	unmethylated	negative	negative	-	-
4	50	9.7	27.2*	unmethylated	negative	n.d.	-	-
5	63	8.6	8.6*	unmethylated	negative	n.d.	-	-
6	60	5.0	14.2*	methylated	negative	n.d.	-	-
7	55	2.6	2.6*	unmethylated	negative	negative	-	-
8	37	16.1	21.4*	methylated	negative	negative	-	-
9	52	8.2	8.2*	methylated	negative	negative	-	-

10	52	2.9	4.5*	unmethylated	n.d.	negative	-	-
11	61	8.0	9.0*	unmethylated	negative	negative	-	-
12	56	7.1	40.2*	methylated	negative	negative	-	-
13	66	23.0	49.7*	methylated	n.d.	negative	n.d.	n.d.
14	47	15.5	15.5*	unmethylated	negative	negative	-	-
15	62	11.4	15.0*	methylated	negative	negative	-	-
16	50	30.3	58.3*	methylated	negative	n.d.	-	-
17	67	7.5	20.9*	methylated	negative	n.d.	-	-
18	45	20.0	33.4*	methylated	negative	negative	-	-
19	61	5.9	8.7*	unmethylated	negative	negative	-	-
20	62	0.5	0.5*	unmethylated	negative	negative	-	-
21	56	3.5	6.0*	methylated	negative	negative	n.d.	n.d.
22	61	12.9	12.9*	unmethylated	negative	negative	-	-
23	53	2.2	10.4*	methylated	negative	negative	n.d.	n.d.
24	55	6.6	6.6*	unmethylated	negative	negative	-	-
25	57	4.8	12.6*	unmethylated	negative	negative	-	-
26	64	4.5	9.6*	unmethylated	negative	negative	-	-
27	72	4.8	13.3*	unmethylated	negative	negative	-	-
28	65	0.7	16.6*	unmethylated	n.d.	negative	n.d.	n.d.
29	71	13.6	13.6*	unmethylated	negative	negative	-	-
30	39	19.6	23.1*	unmethylated	n.d.	negative	n.d.	n.d.
31	54	12.8	19.7*	unmethylated	n.d.	negative	n.d.	n.d.
32	58	8.7	18.7*	methylated	negative	positive	-	-
33	76	6.0	6.0*	methylated	positive	n.d.	-	-
34	59	11.5	11.5*	unmethylated	positive	positive	-	-
35	47	11.2	30.0*	methylated	positive	n.d.	n.d.	n.d.
36	57	6.9	12.9*	unmethylated	positive	n.d.	n.d.	n.d.
37	69	11.6	11.6*	unmethylated	positive	positive	-	-
38	68	6.3	6.3*	unmethylated	positive	positive	-	-
39	37	2.1	2.1*	methylated	positive	positive	-	-
40	69	3.2**	9.1*	methylated	positive	n.d.	-	-
41	62	6.6	16.1*	methylated	positive	n.d.	-	-
42	50	9.9	9.9*	methylated	positive	n.d.	-	-
43	70	0.3	0.3*	methylated	positive	positive	-	-

44	29	13.7	21.9*	methylated	positive	positive	-	-
45	59	9.0	11.2*	unmethylated	positive	positive	-	-
46	56	9.5	21.5*	methylated	positive	positive	-	-
47	56	32.3	39.6*	unmethylated	positive	positive	-	-
48	66	7.5	7.5*	methylated	positive	positive	-	-
49	57	2.3	2.3*	methylated	positive	positive	-	-
50	74	54.0	60.0*	unmethylated	positive	positive	-	-
51	75	5.1	5.1*	unmethylated	negative	positive	-	-
52	54	2.4	2.4*	methylated	positive	positive	-	-
53	68	2.2	2.6*	unmethylated	positive	positive	-	-
54	63	22.4	27.2*	methylated	positive	positive	n.d.	n.d.
55	70	6.7	9.6*	unmethylated	positive	positive	n.d.	n.d.
56	57	2.7	2.7*	unmethylated	negative	positive	-	-
57	76	5.5	10.8*	methylated	negative	positive	-	-
58	48	7.8	16.3*	unmethylated	positive	positive	n.d.	n.d.
59	54	8.3	11.6*	methylated	positive	positive	-	-
60	68	94.6**	94.6	methylated	n.d.	positive	-	-
61	82	2.3	4.2*	methylated	positive	positive	-	-
62	63	7.1	7.1*	unmethylated	positive	positive	-	-
63	65	6.2	6.2*	unmethylated	positive	positive	-	-
64	57	16.6	34.9*	methylated	positive	n.d.	-	-
65	63	7.4	27.5*	methylated	positive	positive	-	-
66	86	3.0	24.9*	methylated	n.d.	positive	n.d.	n.d.
67	67	10.2	17.0*	methylated	negative	negative	negative	negative
68	58	8.7	20.4*	unmethylated	negative	negative	n.d.	negative
69	72	14.3	21.3*	unmethylated	negative	negative	negative	n.d.
70	50	5.2	13.2*	unmethylated	n.d.	negative	n.d.	negative
71	59	5.2 §	23.7*	methylated	n.d.	negative	n.d.	negative
72	52	30.4	54.5*	methylated	negative	negative	n.d.	negative
73	48	40.2	61.9*	methylated	negative	negative	n.d.	negative
74	71	3.7	41.2*	unmethylated	negative	negative	negative	negative
75	71	13.1	23.1*	unmethylated	negative	n.d.	negative	n.d.
76	58	12.0	50.4*	unmethylated	negative	n.d.	negative	n.d.
77	47	5.8	7.5	unmethylated	negative	negative	negative	negative

78	60	7.7	19.4	methylated	negative	negative	negative	negative
79	50	4.6	21.2*	unmethylated	negative	negative	negative	positive
80	62	6.6	26.9	methylated	negative	negative	negative	negative
81	61	3.1	13.9*	unmethylated	negative	negative	negative	negative
82	49	3.3	14.9*	unmethylated	negative	positive	positive	positive
83	54	6.5	11.0*	methylated	positive	positive	positive	positive
84	58	15.1	24.0*	methylated	positive	positive	negative	negative
85	70	3.8	12.3*	methylated	negative	positive	negative	positive
86	64	19.6	24.9*	unmethylated	positive	positive	positive	n.d.
87	63	13.0	17.9*	unmethylated	negative	positive	negative	positive
88	69	7.6	20.1*	methylated	positive	positive	positive	positive
89	57	18.1	26.1*	methylated	positive	positive	n.d.	positive
90	58	8.8	16.8*	unmethylated	negative	positive	n.d.	positive
91	56	21.3	33.2*	methylated	negative	positive	n.d.	positive
92	40	18.1	25.8*	unmethylated	positive	positive	n.d.	positive
93	60	7.0 §	61.0*	methylated	positive	positive	positive	positive
94	58	9.6	16.8*	unmethylated	positive	positive	positive	positive
95	67	3.9	18.8*	unmethylated	positive	positive	negative	n.d.
96	39	18.9	38.2*	unmethylated	positive	positive	negative	negative
97	73	9.2	12.2	unmethylated	positive	positive	positive	positive
98	49	10.0	17.0*	unmethylated	positive	positive	positive	positive
99	64	9.6	10.7	unmethylated	positive	positive	negative	negative
100	75	27.7	32.3*	methylated	positive	positive	positive	positive
101	71	23.9	37.0	unmethylated	positive	n.d.	positive	positive
102	66	32.7	49.2*	unmethylated	positive	n.d.	positive	n.d.
103	55	3.0 §	20.8*	unmethylated	positive	n.d.	positive	n.d.
104	43	6.4	34.1	unmethylated	positive	positive	positive	positive
105	72	26.1	48.4	methylated	positive	positive	positive	positive
106	65	6.1	15.5*	unmethylated	positive	positive	positive	positive

Supplementary Table 2. Overview of individual survival times, *MGMT* promoter methylation status and results of EGFRvIII testing by immunohistochemistry (IHC) or reverse transcription-PCR (RT-PCR) in primary tumors (PT) and recurrent tumors (RT) of 33 patients with newly diagnosed IDH-wildtype, *EGFR*-non-amplified glioblastomas. N.d., not determined.

Patient number	Age (years)	PFS (months)	OS (months)	*MGMT* promoter status	PT EGFRvIII IHC	PT EGFRvIII RT-PCR	RT EGFRvIII IHC	RT EGFRvIII RT-PCR
107	64	10.3	25.3*	methylated	negative	n.d.	negative	n.d.
108	55	2.4	17.9*	methylated	negative	n.d.	negative	n.d.
109	68	3.6	16.7*	unmethylated	n.d.	negative	n.d.	negative
110	75	10.9	13.8*	unmethylated	negative	n.d.	negative	n.d.
111	67	3.7	21.9*	methylated	negative	n.d.	negative	n.d.
112	66	3.3	22.9*	unmethylated	negative	n.d.	negative	n.d.
113	53	42.7	47.5*	methylated	negative	n.d.	negative	n.d.
114	36	5.5	12.2*	unmethylated	negative	n.d.	negative	n.d.
115	33	8.4	13.7*	methylated	negative	n.d.	negative	negative
116	42	3.2	32.5	methylated	negative	n.d.	negative	n.d.
117	59	5.2	15.8	unmethylated	negative	n.d.	negative	n.d.
118	38	7.4	15.5	unmethylated	negative	n.d.	negative	n.d.
119	46	5.5	12.7	methylated	negative	n.d.	negative	n.d.
120	48	3.1	11.9	unmethylated	negative	n.d.	negative	n.d.
121	54	3.4	23.7*	methylated	negative	n.d.	negative	n.d.
122	57	3.8	24.3	unmethylated	negative	n.d.	negative	n.d.
123	61	2.9	16.1*	unmethylated	negative	n.d.	negative	n.d.
124	72	5.9	5.9	unmethylated	negative	n.d.	negative	n.d.
125	72	12.2	41.4	unmethylated	negative	n.d.	negative	n.d.
126	80	3.2	5.2	methylated	negative	n.d.	negative	n.d.
127	47	6.7	16.8	unmethylated	negative	n.d.	negative	n.d.
128	55	4.2	16.4*	unmethylated	negative	n.d.	negative	n.d.
129	53	4.2	14.0*	methylated	negative	n.d.	negative	n.d.

130	69	5.4	12.5	unmethylated	negative	n.d.	negative	n.d.
131	61	11.6	17.4*	unmethylated	negative	n.d.	negative	n.d.
132	57	7.8	79.5*	methylated	negative	n.d.	negative	n.d.
133	60	30.6	83.1*	methylated	negative	n.d.	negative	n.d.
134	58	3.7	16.0*	methylated	negative	n.d.	negative	n.d.
135	67	3.0	15.0*	unmethylated	negative	n.d.	negative	n.d.
136	53	3.1	15.9*	methylated	negative	n.d.	negative	n.d.
137	57	8.1	26.6*	unmethylated	negative	n.d.	negative	n.d.
138	43	12.4	21.7*	unmethylated	negative	n.d.	negative	n.d.
139	63	3.1	14.1*	methylated	negative	n.d.	negative	n.d.

Supplementary Table 3. Overview of the *EGFR* single nucleotide variants (SNVs) detected by next generation sequencing (NGS) in primary (PT) and recurrent (RT) glioblastomas of 27 patients. Mutant allele frequencies in percent are provided for each SNV. References are provided for those SNVs that were already reported by other authors. The table also indicates the presence of *EGFR* amplification as detected by real time-PCR or NGS, and EGFRvIII as detected by NGS or by immunohistochemistry (IMHC) and/or RT-PCR. Note that NGS detected all instances of EGFR amplification, but was less thansensitive compared to IHC?MH and/or RT-PCR in detecting EGFRvIII.

Kommentiert [WM1]: Is this not more common?

Patient number	PT / RT	*EGFR* amplification (real time-PCR)	EGFR amplification (NGS)	EGFRvIII (RT-PCR or IMH)	EGFRvIII (NGS)	*EGFR SNVs* (mutant allele frequencies in %)	References
69	PT	+	+	-	-	c.G1793T:p.G598V (47); c.A2941C:p.I981L (96)	59, 60
	RT	+	+	-	-	c.G1793T:p.G598V (06); c.A2941C:p.I981L (79)	59, 60
79	PT	+	+	-	-	-	
	RT	+	+	+	+	c.C1088T:p.T363I (47)	
83	PT	+	+	+	+	-	
	RT	+	+	+	+	-	
84	PT	+	+	+	-	-	
	RT	+	+	-	-	c.C866T:p.A289V (93)	59, 60
87	PT	+	+	+	-	c.T185G:p.L62R (94); c.G1562A:p.R521K (04); c.G1280A:p.R427H (07)	
	RT	+	+	+	-	c.T185G:p.L62R (07); c.G1562A:p.R521K (07); c.G1280A:p.R427H (22)	
90	PT	+	+	+	+	-	
	RT	+	+	+	+	-	
93	PT	+	+	+	+	c.C866A:p.A289D (11)	14, 59
	RT	+	+	+	-	c.C866A:p.A289D (06)	59
94	PT	+	+	+	-	c.C574G:p.P192A (18)	
	RT	+	+	+	-	c.C574G:p.P192A (06)	
96	PT	+	+	+	-	c.G1784A:p.C595Y (12)	
	RT	+	+	-	-	c.G2303T:p.S768I (04)	
97	PT	+	+	+	+	c.A787C:p.T263P (70)	59
	RT	+	+	+	+	c.A787C:p.T263P (70)	59
98	PT	+	+	+	+	c.G865A:p.A289T (15)	59

	RT	+	+	+	+	-	
99	PT	+	+	+	+	-	
	RT	+	+	-	-	c.C866T:p.A289V (29)	14, 59, 60
100	PT	+	+	+	-	c.G1793T:p.G598V (80)	
	RT	+	+	+	+	-	
108	PT	-	-	-	-	-	
	RT	-	-	-	-	c.G2045A:p.G682D (07): c.G3485Ap.S1162N (06)	
115	PT	-	-	-	-	-	
	RT	-	-	-	-	-	
116	PT	-	-	-	-	-	
	RT	-	-	-	-	c.G1793T:p.G598V (16)	59, 60
117	PT	-	-	-	-	-	
	RT	-	-	-	-	-	
118	PT	-	-	-	-	-	
	RT	-	-	-	-	-	
119	PT	-	-	-	-	-	
	RT	-	-	-	-	-	
120	PT	-	-	-	-	-	
	RT	-	-	-	-	-	
123	PT	-	-	-	-	c.C1787T:p.P596L (13)	59
	RT	-	-	-	-	c.C1787T:p.P596L (10)	59
124	PT	-	-	-	-	-	
	RT	-	-	-	-	-	
125	PT	-	-	-	-	-	
	RT	-	-	-	-	-	
126	PT	-	-	-	-	-	
	RT	-	-	-	-	-	
127	PT	-	-	-	-	-	
	RT	-	-	-	-	-	
135	PT	-	-	-	-	-	
	RT	-	-	-	-	-	
137	PT	-	-	-	-	-	
	RT	-	-	-	-	-	

Supplementary Table 4. Overview of the validation cohort of 150 TCGA patients with IDH-wildtype glioblastoma (GBM). AF, allele frequency; WT, wildtype; M, methylated; UM, unmethylated; NA, not analyzed; TMZ, temozolomide.

Case-ID	Histology	IDH status	*MGMT* promoter status	*EGFR* copy number [GISTIC -score]	*EGFR* SNV	EGFRvIII positive [AF > 0.01]	Age [years]	Gender	Therapy	Survival [months]	Vital status [1: deceased; 0: censored]
TCGA-02-0055	GBM	WT	UM	1	-	NA	62	female	TMZ/radiation→TMZ	2.5	1
TCGA-06-0124	GBM	WT	M	2	-	NA	67	male	Unspecified radiation→TMZ	20.4	1
TCGA-06-0125	GBM	WT	M	2	G598V	NA	63	female	TMZ/radiation→TMZ	47.6	1
TCGA-06-0130	GBM	WT	UM	1	-	NA	54	male	TMZ/radiation→TMZ	12.9	1
TCGA-06-0132	GBM	WT	NA	2	-	NA	49	male	Standard radiation→TMZ	25.3	1
TCGA-06-0139	GBM	WT	UM	1	-	NA	40	male	Standard radiation→TMZ	11.9	1
TCGA-06-0141	GBM	WT	UM	1	-	NA	62	male	TMZ/radiation→TMZ	10.3	1
TCGA-06-0168	GBM	WT	NA	1	-	NA	59	female	TMZ/radiation→TMZ	19.6	1
TCGA-06-0169	GBM	WT	UM	2	-	NA	68	male	TMZ/radiation→TMZ	3.3	1
TCGA-06-0171	GBM	WT	NA	1	-	NA	65	male	Non-standard radiation→TMZ	13.1	1
TCGA-06-0184	GBM	WT	NA	2	-	NA	63	male	Standard radiation→TMZ	40.3	0
TCGA-06-0185	GBM	WT	NA	2	V651M	NA	54	male	TMZ/radiation→TMZ	37.0	0
TCGA-06-0188	GBM	WT	NA	1	-	NA	71	male	Standard radiation→TMZ	28.5	0
TCGA-06-0189	GBM	WT	NA	2	-	NA	55	male	Standard radiation→TMZ	15.4	1
TCGA-06-0190	GBM	WT	NA	1	-	NA	62	male	Standard radiation→TMZ	10.4	1
TCGA-06-0237	GBM	WT	NA	2	L62R	NA	75	female	TMZ/radiation→TMZ	13.6	1
TCGA-06-0238	GBM	WT	NA	1	-	NA	46	male	TMZ/radiation→TMZ	13.3	1
TCGA-08-0386	GBM	WT	NA	2	-	NA	74	male	TMZ/radiation→TMZ	18.0	1
TCGA-12-0615	GBM	WT	NA	2	-	NA	78	female	Non-standard radiation→TMZ	15.3	1
TCGA-12-0616	GBM	WT	NA	2	G598V	NA	36	female	TMZ/radiation→TMZ	14.7	1
TCGA-12-0618	GBM	WT	NA	1	-	NA	49	male	TMZ/radiation→TMZ	13.0	1
TCGA-12-0619	GBM	WT	NA	2	G598V	NA	60	male	TMZ/radiation→TMZ	34.9	1
TCGA-06-0644	GBM	WT	NA	1	-	NA	71	male	Standard radiation→TMZ	12.3	0
TCGA-06-0646	GBM	WT	NA	2	-	NA	60	male	TMZ/radiation→TMZ	5.7	1
TCGA-06-0648	GBM	WT	NA	1	-	NA	77	male	Standard radiation→TMZ	9.8	1
TCGA-06-0650	GBM	WT	UM	2	-	NA	39	female	TMZ/radiation→TMZ	23.6	1
TCGA-06-0686	GBM	WT	NA	2	-	NA	53	male	Standard radiation→TMZ	9.4	0
TCGA-12-0688	GBM	WT	NA	2	-	NA	74	male	Standard radiation→TMZ	26.6	1
TCGA-12-0692	GBM	WT	NA	1	-	NA	75	female	TMZ Chemo	3.6	1
TCGA-15-0742	GBM	WT	NA	2	C636Y	NA	65	male	Non-standard radiation→TMZ	13.8	1

TCGA-06-0744	GBM	WT	NA	2	-	NA	66	male	Standard radiation→TMZ	19.5	0
TCGA-14-0786	GBM	WT	M	2	A289V	NA	50	female	Standard radiation→TMZ	23.0	1
TCGA-14-0789	GBM	WT	M	2	-	NA	54	male	TMZ/radiation→TMZ	11.2	1
TCGA-14-0790	GBM	WT	M	2	A289V	NA	64	female	TMZ/radiation→TMZ	13.8	1
TCGA-06-0875	GBM	WT	UM	1	-	NA	61	female	Standard radiation→TMZ	9.2	0
TCGA-06-0876	GBM	WT	M	2	G598V R108K	NA	72	female	Standard radiation→TMZ	8.9	0
TCGA-06-0878	GBM	WT	UM	2	-	NA	74	male	TMZ/radiation→TMZ	7.2	0
TCGA-06-0879	GBM	WT	M	1	-	NA	52	male	Standard radiation→TMZ	23.0	0
TCGA-06-0881	GBM	WT	UM	2	A289V	NA	50	male	Standard radiation→TMZ	5.8	0
TCGA-06-0882	GBM	WT	UM	1	-	NA	30	male	TMZ/radiation→TMZ	5.4	0
TCGA-06-0939	GBM	WT	UM	1	-	NA	79	female	TMZ/radiation→TMZ	20.9	0
TCGA-26-1439	GBM	WT	UM	2	G598V	NA	63	male	TMZ/radiation→TMZ	13.9	1
TCGA-14-1450	GBM	WT	M	1	-	NA	57	female	TMZ/radiation→TMZ	58.7	0
TCGA-28-1747	GBM	WT	M	2	G598V H304Y	+	44	male	Non-standard radiation→TMZ	2.5	1
TCGA-14-1823	GBM	WT	M	2	A289T	´	58	female	TMZ/radiation→TMZ	17.8	1
TCGA-14-1829	GBM	WT	UM	2	T363I	´	57	male	TMZ/radiation→TMZ	7.2	0
TCGA-27-1830	GBM	WT	UM	1	-	´	57	male	Standard radiation→TMZ	5.1	1
TCGA-27-1831	GBM	WT	UM	2	R108K	´	66	male	TMZ/radiation→TMZ	16.6	1
TCGA-27-1832	GBM	WT	UM	2	-	´	59	female	Non-standard radiation→TMZ	9.9	1
TCGA-27-1833	GBM	WT	UM	2	-	NA	67	female	Standard radiation→TMZ	24.2	1
TCGA-27-1834	GBM	WT	M	1	-	´	56	male	TMZ/radiation→TMZ	40.5	1
TCGA-27-1835	GBM	WT	M	1	-	´	53	female	TMZ/radiation→TMZ	21.3	1
TCGA-27-1836	GBM	WT	M	2	-	NA	33	female	TMZ/radiation→TMZ	30.0	1
TCGA-27-1837	GBM	WT	M	2	-	´	36	male	Standard radiation→TMZ	14.0	1
TCGA-27-1838	GBM	WT	UM	2	A289D	NA	59	female	Standard radiation→TMZ	11.5	1
TCGA-32-1970	GBM	WT	UM	1	A289V	´	59	male	TMZ/radiation→TMZ	15.4	1
TCGA-32-1986	GBM	WT	UM	1	-	NA	68	male	Standard radiation→TMZ	12.7	1
TCGA-32-1991	GBM	WT	UM	2	-	NA	60	male	TMZ/radiation→TMZ	16.9	1
TCGA-02-2470	GBM	WT	UM	1	-	NA	57	male	TMZ/radiation→TMZ	12.9	1
TCGA-02-2485	GBM	WT	UM	1	A289D	´	53	male	TMZ/radiation→TMZ	15.4	0
TCGA-02-2486	GBM	WT	UM	1	-	´	64	male	TMZ/radiation→TMZ	16.2	0
TCGA-32-2491	GBM	WT	UM	1	-	NA	63	male	TMZ/radiation→TMZ	12.2	1
TCGA-32-2494	GBM	WT	M	2	-	NA	58	female	TMZ/radiation→TMZ	20.8	1
TCGA-32-2495	GBM	WT	UM	2	-	NA	59	female	TMZ/radiation→TMZ	15.0	1
TCGA-28-2509	GBM	WT	M	1	-	´	77	female	Standard radiation→TMZ	4.8	0
TCGA-28-2513	GBM	WT	UM	2	-	´	69	female	TMZ/radiation→TMZ	7.3	0

TCGA-28-2514	GBM	WT	UM	2	-	´	45	male	Standard radiation→TMZ	5.3	0
TCGA-27-2518	GBM	WT	UM	2	-	NA	52	male	TMZ/radiation→TMZ	24.7	1
TCGA-27-2519	GBM	WT	UM	1	-	´	48	male	TMZ/radiation→TMZ	10.4	0
TCGA-27-2523	GBM	WT	M	2	-	´	63	male	Standard radiation→TMZ	16.1	1
TCGA-27-2524	GBM	WT	UM	1	-	´	56	male	Standard radiation→TMZ	7.6	1
TCGA-27-2528	GBM	WT	M	2	R222C	´	62	male	TMZ/radiation→TMZ	15.8	1
TCGA-06-2559	GBM	WT	M	1	-	´	83	male	TMZ/radiation→TMZ	4.9	1
TCGA-06-2561	GBM	WT	UM	1	R149W	´	53	female	TMZ/radiation→TMZ	9.3	0
TCGA-06-2562	GBM	WT	UM	1	-	´	81	male	NonStandard radiation→TMZ	2.8	0
TCGA-06-2563	GBM	WT	M	2	R252C	´	72	female	TMZ/radiation→TMZ	8.5	0
TCGA-06-2564	GBM	WT	UM	2	-	+	50	male	Non-standard radiation→TMZ	5.9	0
TCGA-06-2565	GBM	WT	M	2	R108K	´	59	male	TMZ/radiation→TMZ	6.8	0
TCGA-06-2567	GBM	WT	M	1	-	´	65	male	Non-standard radiation→TMZ	4.4	1
TCGA-41-2571	GBM	WT	UM	1	-	´	89	male	TMZ Chemo	0.9	1
TCGA-41-2572	GBM	WT	UM	2	-	´	67	male	TMZ/radiation→TMZ	13.3	1
TCGA-41-2573	GBM	WT	M	1	-	NA	59	male	TMZ/radiation→TMZ	8.9	0
TCGA-41-2575	GBM	WT	UM	2	-	NA	75	male	TMZ/radiation→TMZ	9.5	1
TCGA-32-2615	GBM	WT	UM	2	-	´	62	male	Non-standard radiation→TMZ	15.9	1
TCGA-19-2619	GBM	WT	M	2	-	´	55	female	TMZ/radiation→TMZ	9.7	0
TCGA-19-2620	GBM	WT	M	2	G598V	+	70	male	TMZ/radiation→TMZ	4.9	1
TCGA-19-2623	GBM	WT	M	1	-	NA	65	male	TMZ/radiation→TMZ	7.5	0
TCGA-32-2634	GBM	WT	M	0	-	´	82	male	TMZ/radiation→TMZ	8.9	0
TCGA-32-2638	GBM	WT	M	2	-	´	67	male	TMZ/radiation→TMZ	7.4	0
TCGA-41-3393	GBM	WT	UM	2	-	NA	81	female	TMZ/radiation→TMZ	4.4	1
TCGA-12-3649	GBM	WT	UM	2	-	NA	76	male	TMZ Chemo	15.2	1
TCGA-12-3650	GBM	WT	UM	2	A289V	´	46	male	TMZ/radiation→TMZ	10.9	1
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TCGA-41-3915	GBM	WT	M	1	-	´	48	male	TMZ/radiation→TMZ	6.0	0
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TCGA-76-4925	GBM	WT	M	1	-	´	76	male	NonStandard radiation→TMZ	4.8	1
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TCGA-76-4935	GBM	WT	M	1	-	NA	52	female	Standard radiation→TMZ	10.8	0
TCGA-26-5132	GBM	WT	M	2	-	+	74	male	TMZ/radiation→TMZ	9.4	0

TCGA-26-5133	GBM	WT	UM	0	-	´	59	male	TMZ/radiation→TMZ	14.9	0
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TCGA-28-5207	GBM	WT	UM	1	A289T	´	71	male	NonStandard radiation→TMZ	11.3	1
TCGA-28-5208	GBM	WT	M	1	-	´	52	male	TMZ/radiation→TMZ	15.6	0
TCGA-28-5209	GBM	WT	M	2	G598A	´	66	female	NonStandard radiation→TMZ	2.5	0
TCGA-28-5211	GBM	WT	UM	2	-	NA	42	male	TMZ/radiation→TMZ	11.8	0
TCGA-28-5213	GBM	WT	UM	1	G598V	´	72	male	Standard radiation→TMZ	9.8	0
TCGA-28-5214	GBM	WT	UM	2	-	NA	53	male	NonStandard radiation→TMZ	14.2	0
TCGA-28-5215	GBM	WT	M	1	-	´	62	female	NonStandard radiation→TMZ	11.0	1
TCGA-28-5216	GBM	WT	UM	0	-	´	52	male	TMZ/radiation→TMZ	13.6	0
TCGA-28-5218	GBM	WT	UM	1	-	´	63	male	Standard radiation→TMZ	5.2	1
TCGA-28-5219	GBM	WT	M	1	-	NA	47	female	NonStandard radiation→TMZ	8.5	0
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TCGA-12-5295	GBM	WT	M	2	-	+	60	female	TMZ/radiation→TMZ	14.9	1
TCGA-12-5299	GBM	WT	UM	1	-	´	56	female	TMZ/radiation→TMZ	3.2	1
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TCGA-06-5408	GBM	WT	UM	2	-	+	54	female	Standard radiation→TMZ	11.7	1
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TCGA-06-5413	GBM	WT	UM	2	-	´	67	male	Standard radiation→TMZ	8.8	0
TCGA-06-5414	GBM	WT	UM	2	-	+	61	male	TMZ/radiation→TMZ	9.0	0
TCGA-06-5415	GBM	WT	UM	2	-	+	60	male	TMZ/radiation→TMZ	8.5	0
TCGA-41-5651	GBM	WT	M	1	-	´	59	female	TMZ/radiation→TMZ	11.5	0
TCGA-06-5858	GBM	WT	UM	1	-	´	45	female	TMZ/radiation→TMZ	6.1	0
TCGA-06-5859	GBM	WT	UM	1	-	´	63	male	TMZ/radiation→TMZ	4.6	0
TCGA-19-5950	GBM	WT	M	2	-	NA	52	female	TMZ/radiation→TMZ	11.3	0
TCGA-19-5951	GBM	WT	UM	2	-	NA	76	female	TMZ/radiation→TMZ	8.0	1
TCGA-19-5954	GBM	WT	M	2	-	NA	72	female	TMZ/radiation→TMZ	8.0	0
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TCGA-19-5960	GBM	WT	UM	1	-	´	56	male	TMZ/radiation→TMZ	5.4	0
TCGA-26-6173	GBM	WT	UM	2	-	NA	57	male	TMZ/radiation→TMZ	7.9	0
TCGA-26-6174	GBM	WT	M	1	-	NA	65	female	TMZ/radiation→TMZ	2.3	0
TCGA-76-6191	GBM	WT	UM	1	C620W	NA	57	male	TMZ/radiation→TMZ	16.7	1
TCGA-76-6192	GBM	WT	UM	1	-	NA	74	male	Standard radiation→TMZ	3.3	1
TCGA-76-6193	GBM	WT	UM	0	-	NA	78	male	Standard radiation→TMZ	2.7	1
TCGA-76-6282	GBM	WT	UM	2	C571S	NA	63	male	TMZ/radiation→TMZ	17.1	1

TCGA-76-6285	GBM	WT	UM	1	-	NA	64	female	TMZ Chemo	8.3	1
TCGA-06-6388	GBM	WT	UM	1	-	NA	64	female	TMZ/radiation→TMZ	5.2	1
TCGA-06-6390	GBM	WT	UM	1	-	NA	58	male	TMZ/radiation→TMZ	5.4	1
TCGA-28-6450	GBM	WT	UM	2	-	NA	60	male	TMZ/radiation→TMZ	5.4	1
TCGA-74-6573	GBM	WT	UM	2	-	NA	67	male	TMZ/radiation→TMZ	3.4	1
TCGA-06-6695	GBM	WT	M	1	-	NA	64	male	TMZ/radiation→TMZ	8.3	0
TCGA-06-6698	GBM	WT	UM	1	-	NA	53	female	Non-standard radiation→TMZ	4.8	0
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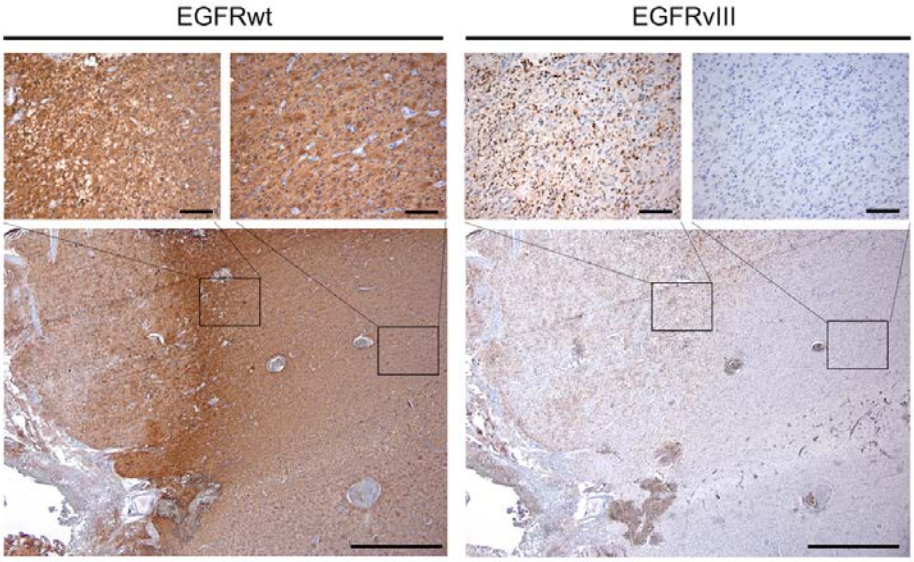
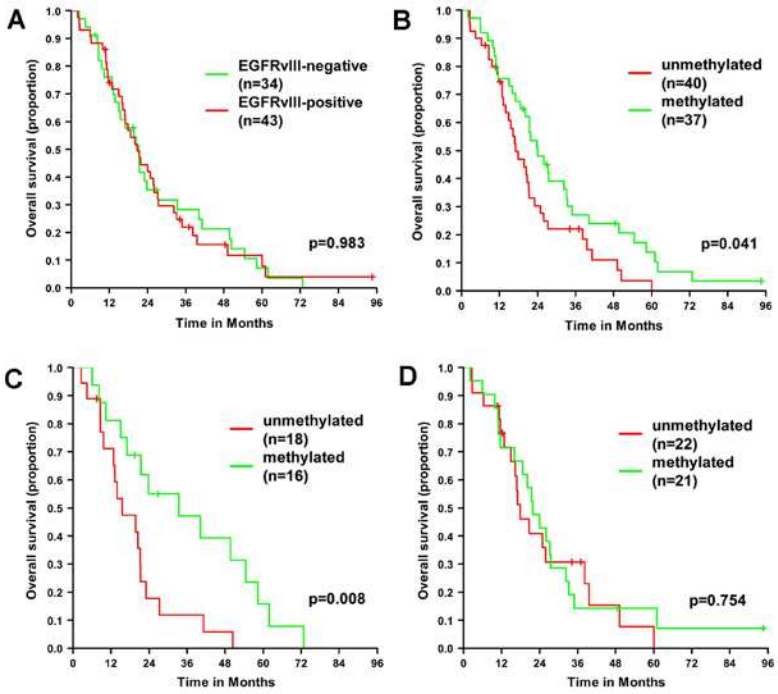
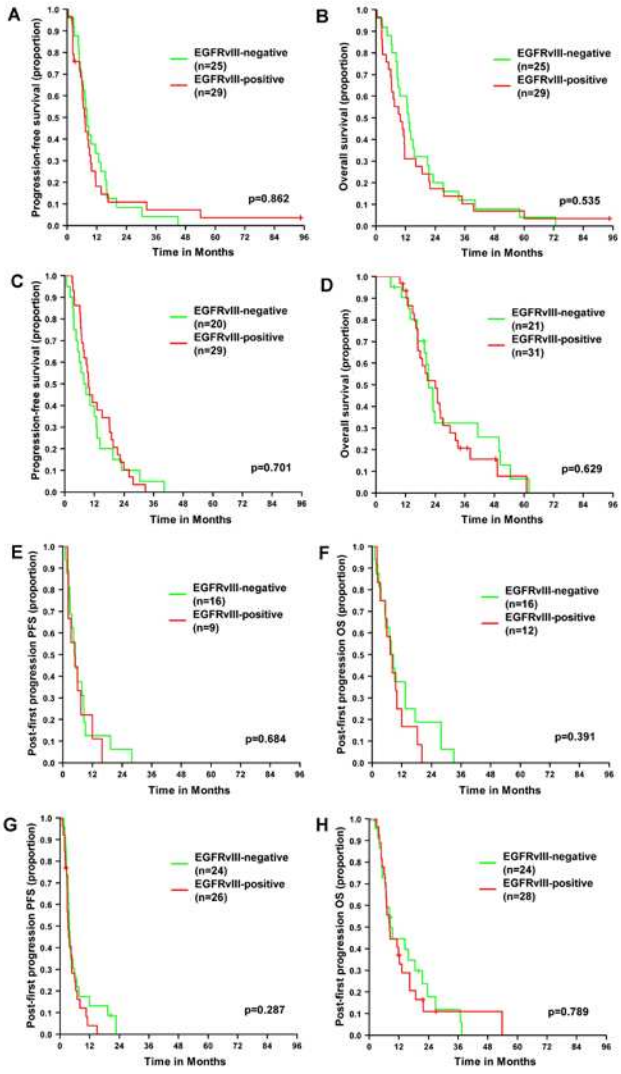
Supplementary Figures



Supplementary Figure 1. Demonstration of regional heterogeneity of EGFRvIII expression in an EGFR-amplified primary glioblastoma, IDH-wildtype (patient 96). Shown are consecutive sections immunstained with antibodies against EGFR wildtype (EGFRwt, left side) and EGFRvIII (EGFRwt, right side). Note that EGFRwt expression is homogeneously distributed across the entire tumor specimen. In contrast, EGFRvIII-positivity is restricted to a subpopulation of tumor cells in the left upper area of the tumor while the tumor parts on the right-hand side are mostly negative for EGFRvIII. Sections are counterstained with hemalum. Scale bars correspond to 50 μm in the high magnification images at the top and 500 μm in the low magnification images at the bottom.



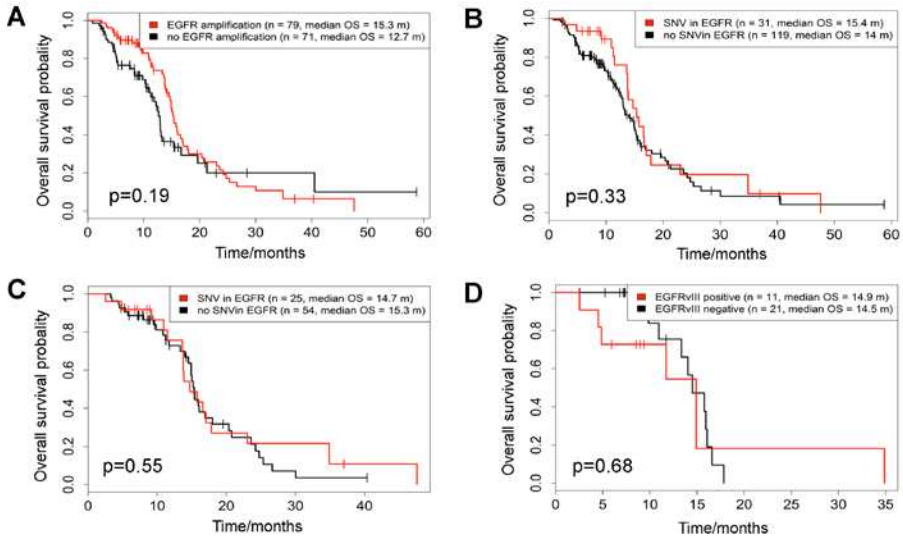
Supplementary Figure 2. EGFRvIII expression and *MGMT* promoter methylation status and survival outcome in 77 patients with *EGFR*-amplified glioblastomas treated with radiotherapy and temozolomide chemotherapy. **(A)** Overall survival (OS) in the 77 patients stratified according to EGFRvIII status. **(B)** OS in the 77 patients stratified according to *MGMT* promoter methylation status. **(C)** OS in the subgroup of patients with EGFRvIII-negative tumors stratified according to *MGMT* promoter methylation status. **(D)** OS in the subgroup of patients with EGFRvIII-positive tumors stratified according to *MGMT* promoter methylation status.



Supplementary Figure 3. Survival of patients with *EGFR*-amplified glioblastomas, IDH-wildtype, according to the EGFRvIII status in the subgroups of patients who received second surgery (52 patients) or did not receive second surgery (54 patients). (**A)** PFS and **(B)** OS in patients who did not receive second surgery. **(C)** PFS and (**D)** OS in patients who received second surgery. Post-first progression PFS and OS from first progression in patients who were not re-operated **(E,F)** or were re-operated **(G,H)**.

Fehlt noch aus Leipzig

Supplementary Figure 4. Survival of 27 German Glioma Network patients with glioblastoma, IDH-wildtype, according to presence (14 patients) or absence (13 patients) of at least one *EGFR* single nucleotide variant (SNV) as detected by NGS~~next generation sequencing~~ of the *EGFR* coding sequence.



Supplementary Figure 5. Survival of TCGA patients with glioblastoma, IDH-wildtype, according to *EGFR* amplification, presence of at least one *EGFR* single nucleotide variant (SNV), or EGFRvIII positivity. **(A)** OS in 150 TCGA patients with glioblastoma, IDH-wildtype, stratified according to presence (79 patients) or absence (71 patients) of *EGFR* ~~gene~~ amplification *EGFR* SNVs. **(B)** OS in 150 TCGA patients with glioblastoma, IDH-wildtype, stratified according to presence (31 patients) or absence (119 patients) of at least one *EGFR* SNV. **(C)** OS in 79 TCGA patients with *EGFR*-amplified glioblastoma, IDH-wildtype, stratified according to presence (25 patients) or absence (54 patients) of at least one *EGFR* SNV. **(D)** OS in 33 TCG patients with *EGFR*-amplified glioblastoma, IDH-wildtype, stratified according to presence (11 patients) or absence (21 patients) of EGFRvIII posiivity. Note that neither *EGFR* amplification nor presence of *EGFR* SNVs were associated with OS in the entire patient cohort (A-B). Within the subgroup of EGFR-amplified glioblastoma patients, neither presence of *EGFR* SNVs nor EGFRvIII were associated with~~to~~ OS (C-D).

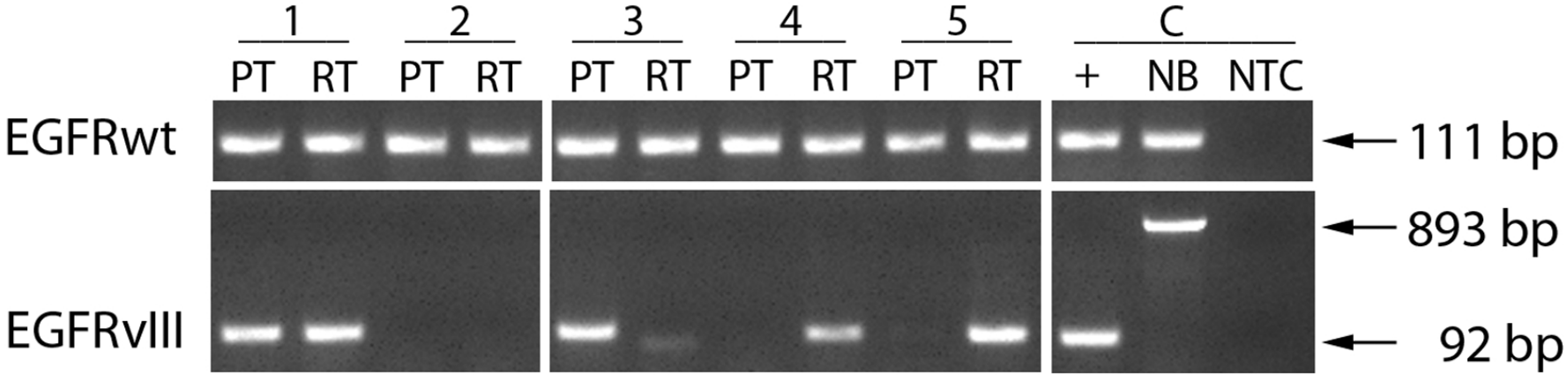
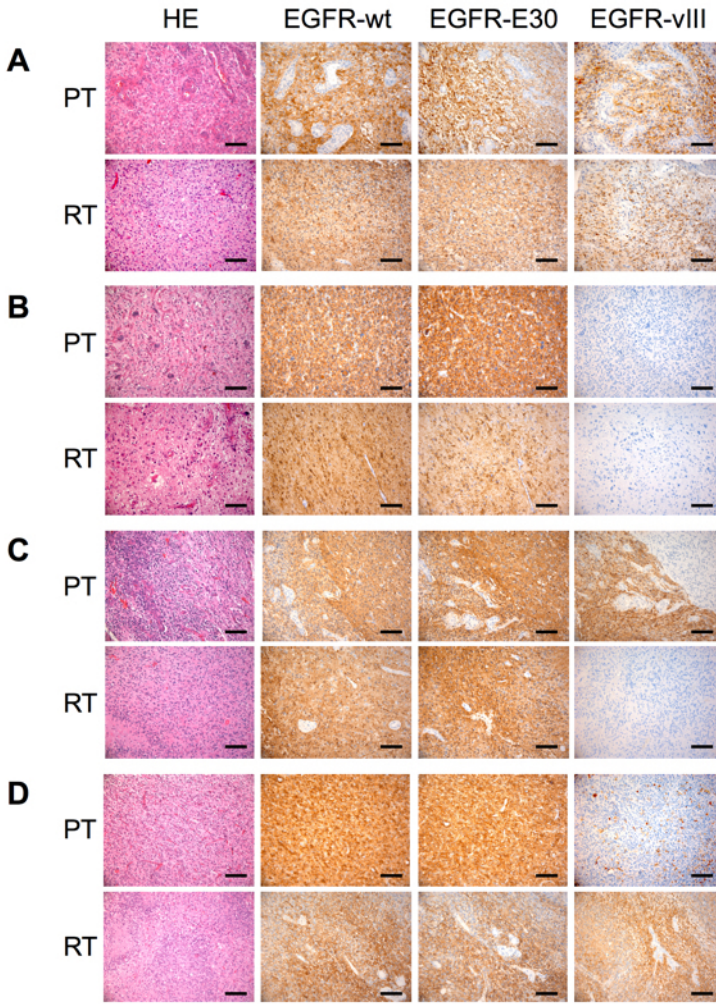
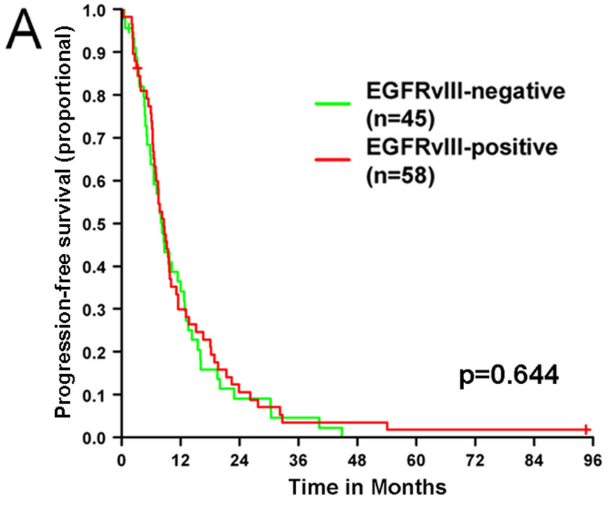
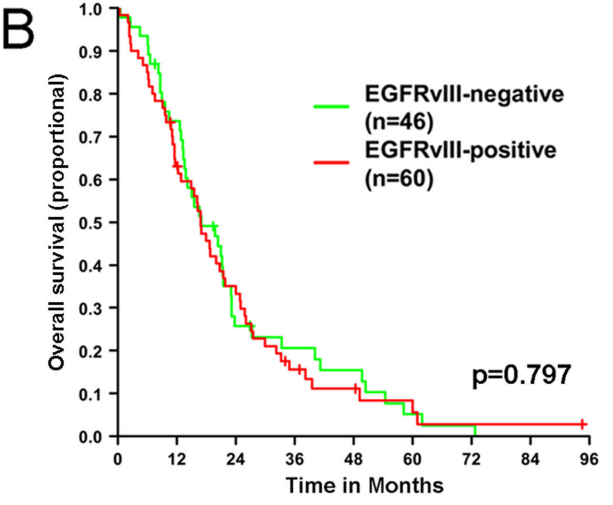
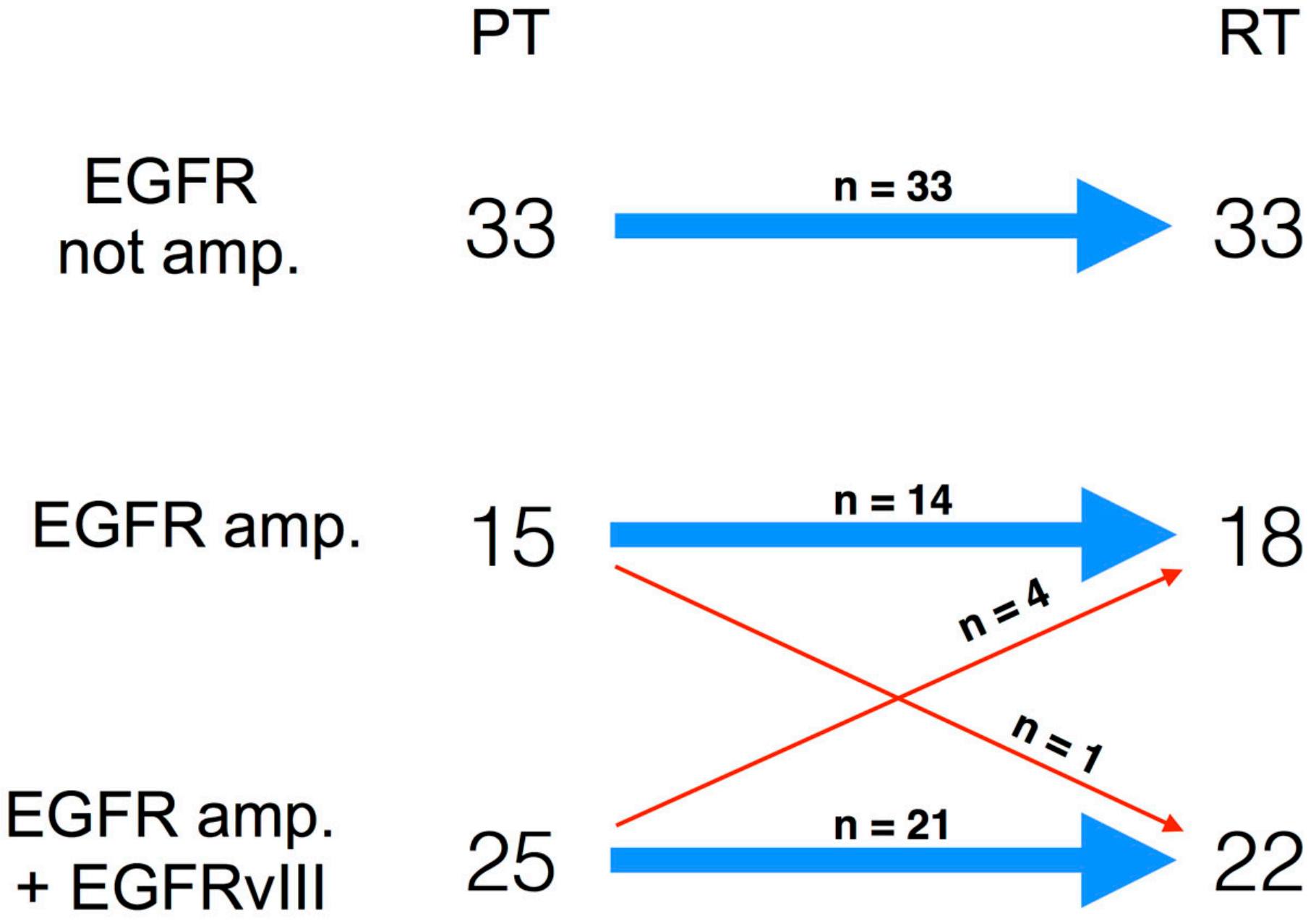

1
2
3
4
5
C
PT RT PT RT PT RT PT RT PT RT + NB NTC
EGFRwt
EGFRvIII
111 bp
893 bp
92 bp

Fig. 2




A
Progression-free survival (proportional)
1.0
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0.0
0
12
24
36
48
60
72
84
96
Time in Months
EGFRvIII-negative
(n=45)
EGFRvIII-positive
(n=58)
p=0.644


B
Overall survival (proportional)
1.0
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0.0
0
12
24
36
48
60
72
84
96
Time in Months
EGFRvIII-negative
(n=46)
EGFRvIII-positive
(n=60)
p=0.797


PT
RT
EGFR
not amp.
33
n = 33
33
EGFR amp.
15
n = 14
18
n = 4
n = 1
EGFR amp.
+ EGFRvIII
25
n = 21
22